Biochimica et Biophysica Acta 1823 (2012) 1208–1216

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Localization of Ras signaling complex in budding yeast

Fiorella Belotti [1], Renata Tisi [1], Chiara Paiardi [2], Marco Rigamonti, Silvia Groppi, Enzo Martegani *

Dept. of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy

ARTICLE INFO

Article history:
Received 13 September 2011
Received in revised form 26 April 2012
Accepted 30 April 2012
Available online 7 May 2012

Keywords:
Saccharomyces cerevisiae
Cdc25
Ira
Adenylate cyclase
Membrane fractionation
Mitochondria

ABSTRACT

In *Saccharomyces cerevisiae*, cAMP/pKA pathway plays a major role in metabolism, stress resistance and proliferation control. cAMP is produced by adenylate cyclase, which is activated both by Gpr1/Gpa2 system and Ras proteins, regulated by Cdc25/Sdc25 guanine exchange factors and Ira GTPase activator proteins.
Recently, both Ras2 and Cdc25 RasGEF were reported to localize not only in plasma membrane but also in internal membranes. Here, the subcellular localization of Ras signaling complex proteins was investigated both by fluorescent tagging and by biochemical cell membrane fractionation on sucrose gradients. Although a consistent minor fraction of Ras signaling complex components was found in plasma membrane during exponential growth on glucose, Cdc25 appears to localize mainly on ER membranes, while Ira2 and Cyr1 are also significantly present on mitochondria. Moreover, PKA Tpk1 catalytic subunit overexpression induces Ira2 protein to move from mitochondria to ER membranes. These data confirm the hypothesis that different branches of Ras signaling pathways could involve different subcellular compartments, and that relocalization of Ras signaling complex components is subject to PKA control.

1. Introduction

In *Saccharomyces cerevisiae*, cAMP regulates PKA activity, which is involved in post-translational regulation of a variety of proteins, for example key enzymes for gluconeogenesis or glycolysis [1], is required for G1 to S phase progression in the cell cycle and modulates the critical cell size required for budding and for entry into mitosis [2]. Cyclic AMP is synthesized by Cyr1 adenylate cyclase, which is controlled by two different systems: the G-protein-coupled receptor system (GPCR), acting through Gpa2 G-protein [3,4], and Ras small G protein system [5]. Ras protein activity is controlled by regulatory proteins: Cdc25 and Sdc25 guanine nucleotide exchange factors (GEFs) [6], which stimulate the GDP/GTP exchange on Ras, and Ira1 and Ira2 GTPase activating proteins (GAPs), which promote the intrinsically low Ras GTPase activity [7].

Literature data suggest that upstream components of cAMP/PKA pathway interact to constitute a large multiprotein complex: after detergent extraction, adenylate cyclase behaves as a large complex on gel filtration and sucrose density centrifugation [8–11]; consistently, adenylate cyclase interacts with Ras proteins [12,13], and with Cdc25 SH3 motif [14]; moreover, Ira1 was shown to be involved in Cyr1 association to membranes [8,15].

Cdc25 and Ira proteins were shown to be tightly attached to membrane fraction [16,17], adenylate cyclase is only loosely bound to the membranes, migrates in lower density internal membrane fractions on a sucrose gradient [18] and seems to rely on Ira1 for its membrane targeting [8,15].

Recent data have shown that mammalian Ras isoforms are differentially distributed within cell surface nanoclusters and on endomembranous compartments [19]. The three abundant mammalian isoforms – H-Ras, N-Ras and K-Ras4B – have a high degree of sequence identity in their G-domain, the structural element required for the switch function and for effector binding; the differences among the isoform localization and function can be attributed to different post-translational lipid modifications that are encoded within their divergent C termini. In yeast, Ras2 localization on internal membranes was also investigated: Ras proteins are synthesized as cytosolic precursors and then they are anchored into membranes *via* post-translational modifications; most of the Ras modification steps occur on the cytoplasmatic surface of the endoplasmic reticulum, Ras2 is engaged to the ER membrane by Eri1 protein [20] and the subsequent Ras translocation from ER to plasma membrane doesn't require the classical secretory pathway in yeast [21,22]. Besides, recent works point to a possible role for mitochondria in Ras localization [23]. Moreover, we have reported that Cdc25 and Ira1 are found not only associated with membranes, but also in the nucleus [24,25], and active Ras was also detected in the nuclear compartment, even if the meaning of this result is still under investigation [26]. Finally, we recently reported that PKA activity is not only able to negatively control Cdc25 localization in the plasma membrane, but

Abbreviations: cAMP, 3′-5′-cyclic adenosine monophosphate; GEF, guanine exchange factor; GAP, GTPase activating protein; ER, endoplasmic reticulum; PKA, protein kinase A; GPCR, G-protein coupled receptor

* Corresponding author at: P.za della Scienza 2, 20126 Milan, Italy. Tel.: +39 02 6448 3533; fax: +39 02 6448 3565.
E-mail address: enzo.martegani@unimib.it (E. Martegani).
[1] These two authors contributed equally to the work.
[2] Present address: Dept. of Biology, University of Milan, Milan, Italy.

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2012.04.016

also in the nuclear compartment [25]. Ras localization was also recently found to be affected by PKA activity [25,27].

Consequently, even if the complex has been initially thought to function at the plasma membrane level, only Ras2 showed a clear, but partial, plasma membrane localization, while no clear data are available for the localization of the proteins involved in this pathway. Here we demonstrate that, during exponential growth on glucose, Ras2 signaling complex components are associated mostly to internal membranes, in particular to ER membranes, suggesting the presence of a large signaling complex working inside the cell rather than on its surface, at least during exponential growth on glucose. In addition a possible role of mitochondria in Ras signaling is reinforced by our observation on the partial localization of both Cyr1 and even more of Ira2 on these organelles.

2. Materials and methods

2.1. Strains and cultures

Top10 *E. coli* strain (Invitrogen) was grown in LB medium (1% NaCl, 1% Bacto-peptone, 0.5% yeast extract, 0.1% glucose) at 37 °C. For Amp^R selection, ampicillin was added to a final concentration of 50 mg/L. For solid media, 1.5% agar was added.

Yeast strains utilized in this work are described in Table 1. Yeast cells were grown in YP (1% yeast extract, 2% Bacto-peptone) supplemented with 2% w/v glucose (YPD). Yeast strains carrying a plasmid were grown in synthetic medium (SC) containing 0.67% w/v yeast nitrogen base w/o amino acids, CSM synthetic amino acid mixture (BIO101, USA) required and the appropriate sugar (2% glucose or 2% galactose with 0.1% glucose). Solid medium contained 2–2.5% agar. In all conditions cells were grown at 30 °C. Cell density was determined by measuring optical density (OD_{600}) or by Coulter Counter (Coulter Electronics mod. Z2). Budding index (BI) was determined by direct microscopic count of at least 300 cells fixed in 4% formalin and mildly sonicated. Cell volume distributions were determined with a Coulter Counter Channelyzer 256 [2].

Cells starved for nutrients or for carbon source were grown in YPD or in SC until exponential phase, then collected by filtration, washed with water, resuspended in 0.25 M MES pH 6.5 and incubated for 4 h.

2.2. Strains and plasmid construction

Strains carrying 4-HA tagged Ira1 and Ira2 were constructed according to the method first described by Wach et al. [28], by transforming the W303-1A strain with a PCR generated cassette amplified from pDHA plasmid [29] (kind gift from P. Coccetti, University of Milan-Bicocca) with Pfu Turbo enzyme (Promega) using as primers: for *IRA1* cassette, IRA1HA FOR and IRA1HA REV; for *IRA2* cassette, IRA2HA FOR and IRA2HA REV (Table 2).

YIplac204T/CSec7-7xDsRed, YIplac204T/CHmg1CFP [30] and YIplac204/TKC-DsRED-HDEL [31] were kind gifts from B. Glick (University of Chicago, USA).

The YIplac204T/CHmg1dsRED plasmid was obtained by substituting the *NcoI–PvuII* fragment in YIplac204T/CHmg1CFP with the *NcoI–HincII* fragment from pDsREDMonomer1 (Clontech).

The pYX-TPI-IRA2 plasmid was created by inserting the 506 bp *EcoRV–SalI* fragment from pd46 plasmid [7] (kind gift from JF. Cannon, University of Missouri, USA) in *XhoI–SmaI* digested pYX012 (Ingenius). The plasmid was linearized with *BglII* and inserted at *IRA2 locus* in YOL081W strain (Invitrogen) giving RT1200 strain.

Diploid RT1220 and RT1210 strains were obtained by conjugation of W303-1B transformed with YIplac204T/CSec7-7xDsRed respectively with YJL005W or RT1200, diploid RT1230 and RT1240 strains were obtained by conjugation of W303-1B transformed with YIplac204T/CHmg1xDsRed respectively with YJL005W or RT1200. RT1211 and RT1221 aploid strains were obtained by sporulation respectively of RT1210 and RT1220 strains, and isolated by random spore analysis.

pYX212-EGFP-RBD-3 and YEpTPK1 plasmids were previously described [26,32].

2.3. Cellular membrane fractionation

The method was first described by Sidoux-Walter et al. [33], with modifications. Cells were grown until mid-log phase (OD_{600} 0.6–1.0) at 30 °C in YPD medium, harvested by centrifugation at 4 °C for 10–15 min, washed with 20 ml of Cell Wash Buffer (CWB) (10 mM Tris–HCl pH 7.5, 0.5 M sucrose, and either 2.5 mM EDTA or 2 mM magnesium chloride, as indicated) and collected by centrifugation. Cell pellet was weighed and frozen at −80 °C.

Table 1
Strains used in this study.

Strain	Relevant genotype	Source
W303-1A	*MAT a ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[58]
W303-1B	*MAT α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[58]
OL568-1C	W303-1A *3xHA-CDC25*	[59]
RT1170	W303-1A *IRA1-4xHA::KanMX6*	This work
RT1180	W303-1A *IRA2-4xHA::KanMX6*	This work
YLR378C	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SEC61-GFP::Sp his5*$^+$	Invitrogen
YEL036C	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ANP1-GFP:: Sp his5*$^+$	Invitrogen
YJL005W	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CYR1-GFP:: Sp his5*$^+$	Invitrogen
YOL081W	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 IRA2-GFP::Sp his5*$^+$	Invitrogen
W303-$CDC25^{NES}$-eGFP	W303-1A [YEp$CDC25^{NES}$-eGFP]	[24]
RT1200	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$	This work
RT1210	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$*/IRA2 trp1-1::YIplac204T/C SEC7-7xDsRed/TRP1*	This work
RT1220	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 CYR1-GFP::Sp his5*$^+$*/CYR1 trp1-1::YIplac204T/C SEC7-7xDsRed/TRP1*	This work
RT1230	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$*/IRA2 trp1-1::YIplac204T/C HMG1-DsRed/TRP1*	This work
RT1240	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 CYR1-GFP::Sp his5*$^+$*/CYR1 trp1-1::YIplac204T/C HMG1-DsRed/TRP1*	This work
RT1211	*MAT a ade2-1 his3 leu2 met15 ura3 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$ *trp1-1*	This work
RT1221	*MAT a ade2-1 his3 leu2 met15 ura3 cyr1::CYR1-GFP::Sp his5*$^+$ *trp1-1*	This work
W303-$CDC25^{NES}$	OL568-1C with *3xHA::CDC25::NES::Sphis5*$^+$	[24]
*cdc25*Δ YEpTPK1	W303-1A *cdc25::HIS3* [YEpTPK1]	[32]

Frozen cells were thawed at 30 °C, washed with 20 ml of Homogenization Buffer (HB) (50 mM Tris–HCl pH 7.5, 0.3 M sucrose, 2.5 mg/ml BSA, and either 5 mM EDTA and 1 mM EGTA or 2 mM magnesium chloride, as indicated), harvested by centrifugation, resuspended in HB (supplemented with 2 mM DTT, 1 mM PMSF, 2 μM pepstatin, Roche EDTA-free protease inhibitor cocktail) with approximately 2 ml of buffer per gram of cells wet-weight. Cells were disrupted with Fast-prep (Savant) in the presence of glass beads.

Extracts were clarified at 13,000 rpm for 10 min, then centrifuged at 100,000 ×*g* for 60 min and the supernatant was stored at −80 °C with 20% glycerol. Pellet (Microsomal Membrane pellet or MMP) was washed with Membrane Wash Buffer (MWB, 10 mM Tris–HCl pH 7, 1 mM DTT, 20%, glycerol and either 1 mM EGTA or 2 mM magnesium chloride, as indicated) and then centrifuged for 60 min at 100,000 ×*g*. Washed MMP was resuspended with 0.5 ml of MWB and overlayed on a 11 ml-sucrose gradient, prepared in sucrose gradient buffer (10 mM Tris–HCl pH 7, 1 mM DTT, and either 1 mM EDTA or 2 mM magnesium chloride, as indicated), with the following steps: 1 ml 46%, 2 ml 50%, 2 ml 54%, 2 ml 56%, 2 ml 58%, 1.7 ml 70% sucrose. Gradients were then centrifuged at 80,000 ×*g* for 17 h, and 1 ml-fractions were collected and stored at −20 °C.

Protein concentration was determined with Bio-Rad protein assay (Bio-Rad) and proteins were separated by SDS-PAGE and visualized by Western blot.

Several protein markers for the intracellular membranes were used, and each fraction was tested for the presence of: Gas1, which is post-translationally modified in ER and Golgi compartments and then is transported to the plasma membrane level [34], although it is present in traces also in mitochondria and nuclear envelope [35,36]; Anp1 as a marker for Golgi apparatus [37], Tom40 for mitochondria [38], Pho8 for vacuolar membranes [39], nucleolar protein Nop1 for nuclear membranes [40] and mature Pma1 for plasma membrane [41], although it also marginally localizes to vacuoles [36,42]. As ER marker Sec61–GFP fusion protein was used and expressed in YLR378C strain (Invitrogen). Protein profile was analyzed by Western blot using the following antibodies: goat α-Ras2 (Santa Cruz), mouse α-HA (Roche), goat α-Cyr1 (Santa Cruz), rabbit BD Living Colors antibody against eGFP protein (BD Biosciences, Clontech), rabbit α-Gas1 (kind gift from M. Vai, University of Milano-Bicocca), rabbit α-Tom40 (kind gift from T. Endo, Nagoya University, Japan), mouse α-Pma1 (Abcam), rabbit α-Anp1 (kind gift from S. Munro, MRC Lab of Molecular Biology, Cambridge, UK), mouse α-Pho8 (kind gift from J. Winderickx, K.U. Leuven, Belgium), mouse α-Nop1 (EnCor Biotechnologies). Secondary horseradish peroxidase-conjugated antibodies were from Jackson Immunoresearch.

2.4. GDP/GTP loading on Ras2

Gradient fractions 1–5 were collected (80–100 μg) and diluted 1:2 with Shalloway buffer 2× (50 mM Hepes pH 7.5, 300 mM NaCl, 20% glycerol, 50 mM NaF, 20 mM $MgCl_2$, 2 mM EDTA, 2 mM Na-vanadate, Roche EDTA-free protease inhibitor cocktail). They were incubated with 1 mM GDP or GTP (Sigma Aldrich) at room temperature for 15 min and then Ras2-GTP/total Ras2 ratio was measured as described by Colombo et al. [32]; briefly, fractions were incubated for 1 h at 4 °C with GST–RBD pre-bound to glutathione–Sepharose and Ras2-GTP bound to GST–RBD protein was eluted with SDS sample buffer (50 mM Tris–HCl pH 6.8, 2.3% SDS, 5% β-mercaptoethanol). Ras2 was detected by Western blot using anti Ras2 antibodies (Santa Cruz Biotechnology).

2.5. Epifluorescent microscopy

Cells were grown at 30 °C in the proper selective SC medium supplemented with 2% glucose to early exponential phase and images were collected and processed as previously described [24].

Cells carrying constructs expressing GFP or DsRed fusion proteins, eventually stained with DAPI, were observed with a Nikon Eclipse C600 fluorescence microscope using standard UV, FITC and Rhodamine filters. Images were acquired with a Leica DG350F CCD camera and elaborated with MacBiophotonics ImageJ software. Panels were mounted with Adobe Photoshop™.

2.6. Confocal microscopy

Confocal microscopy images were collected using the Leica TCS SP2 confocal microscope equipped with an inverted Leica DMIRE2 microscope and a PL APO 63× oil immersion objective (numerical aperture: 1.4). An average of 10 optical sections was acquired for every single cell, and a representative single optical section is shown. Mitochondria staining was realized with 100 nM Rhodamine B, hexyl ester, perchlorate (Molecular Probes). Images were elaborated with MacBiophotonics ImageJ software.

3. Results

Even if the cAMP/PKA pathway has been extensively studied, the physiological and functional localization of Ras signaling complex components is still in debate. No evident signals are detected by different prediction softwares (such as BaCelLo [43] or TargetP [44]) in these proteins for localization in any subcellular compartment such as ER, Golgi apparatus or mitochondria. To investigate this topic in more details, tagging with eGFP or DsRED fluorescent proteins was performed.

Since Cdc25 was previously shown to be efficiently imported and retained in the nuclear compartment [24], fluorescence tagging with eGFP was performed in the W303-1A-CDC25^{NES}-eGFP strain, carrying the Cdc25^{NES} protein, a version of Cdc25 which was modified by the insertion of a strong Nuclear Export Signal, and is therefore excluded from the nucleus. This allowed to better observe the localization of the protein within the cytosolic compartment, without the interference of the intense fluorescence in the nucleus observed for the wild-type Cdc25 protein. This mutant version confirmed that, as it was already observed for the wild-type version [24], Cdc25 is not homogeneously distributed in the cytosol: the co-localization with Hmg1DsRed (a protein localizing to ER when overexpressed) (Fig. 1A) reveals a partial co-localization with this compartment, while a co-localization with Sec7DsRed (a protein partially soluble and partially associated to the Golgi vesicles [36,45]) (Fig. 1B) followed by observation with a confocal microscope, revealed no co-localization in any of the observed cells. The co-staining of mitochondria with Rhodamine B (Fig. 1C) revealed only occasional co-localization with Cdc25eGFP, suggesting that Cdc25 cannot be observed in these compartments.

The localization of other Ras signaling complex components, Cyr1 and Ira2, was then considered. Again, these proteins were too lowly expressed to allow direct immunofluorescence observation. Both the proteins were modified by tagging with GFP, and Ira2-GFP fusion was slightly overexpressed (as revealed by the mild increase in the associated fluorescent signal) by inserting constitutive TPI promoter immediately upstream the Ira2 encoding sequence. These modifications allowed to observe that Ira2 showed significant and reproducible co-localization mainly with mitochondria, stained with DAPI (detectable in all the observed cells), and endoplasmic reticulum (ER), visualized by Hmg1 protein (detectable in 72% of the observed cells), but never with the Golgi marker Sec7 (Fig. 2B). Cyr1 was also partially observed in mitochondria (co-localization with DAPI stained mitochondria was observed in 69% of the observed cells), but it was clearly present in an internal compartment identified as ER (Fig. 2A) (co-localization with Hmg1DsRed fluorescence was observed in 88% of the cells observed). As well as for Cdc25, no co-localization was observed for Cyr1-GFP fusion with the Golgi marker Sec7 (Fig. 2B).

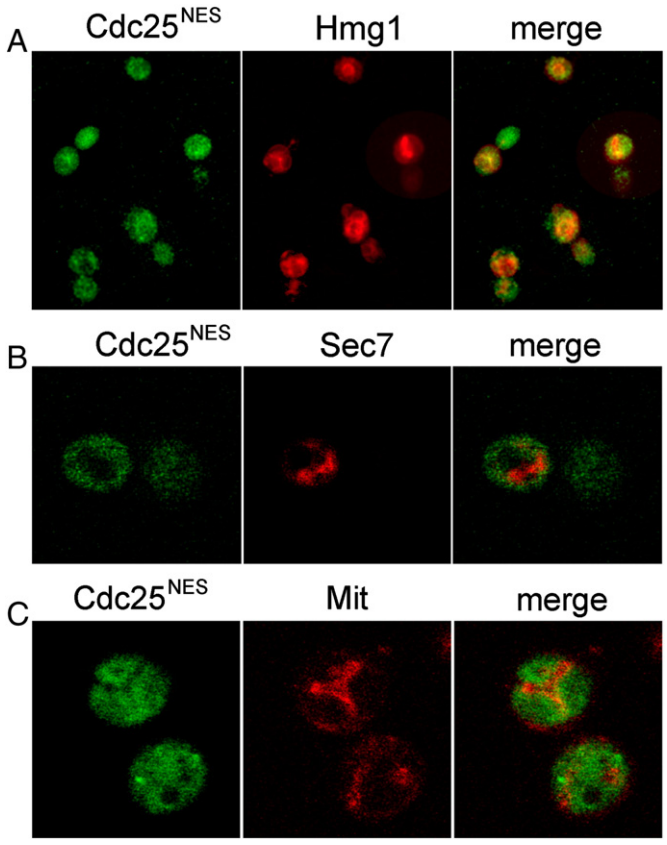

Fig. 1. Cdc25 protein is present on ER internal membranes. W303-CDC25^{NES}eGFP cells transformed either with YIplac204T/CHmg1-DsRed (expressing Hmg1-DsRED, localizing to the ER) (panel A) or with YIplac204T/CSec7-7xDsRed (expressing Sec7-DsRed, localizing to the Golgi apparatus) (panel B), or stained with the Rhodamine B dye for mitochondria (panel C) were observed using a fluorescence (A) or a confocal (B and C) microscope.

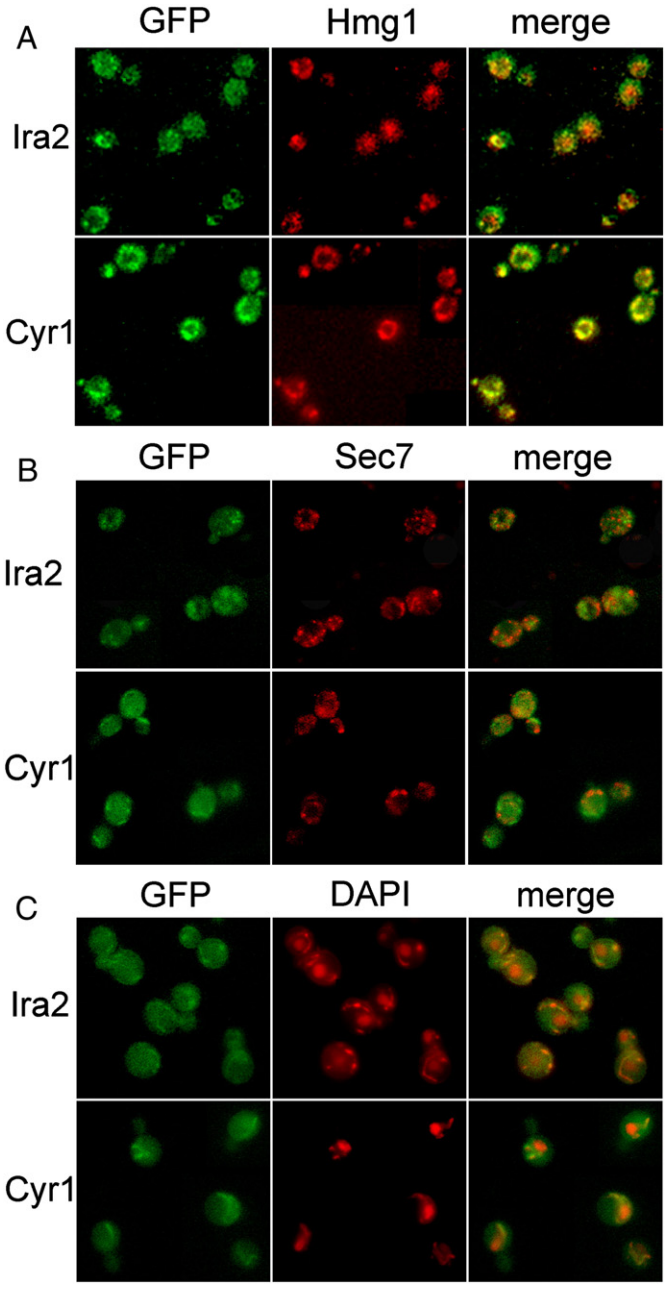

Fig. 2. Ira2 and Cyr1 proteins are present on mitochondrial membranes. RT1230 or RT1210 strains (for Ira2GFP) and RT1240 or RT1220 strains (for Cyr1GFP) were observed using a fluorescence microscope: panel A, colocalization with ER marker Hmg1 is shown; panel B, colocalization with Golgi marker Sec7; panel C, co-localization with DAPI-stained nuclei and mitochondria is shown.

Protein localization was confirmed by performing a cell extract fractionation assay on discontinuous sucrose gradients, followed by the analysis of the distribution of endogenously expressed proteins, in order to reveal any unphysiological effect on protein localization. In fact, mislocalization of Ras was already reported, due to its over-expression and/or fluorescent tagging [23].

Briefly, crude cellular membranes were layered on a 6 steps sucrose gradient (46%, 50%, 54%, 56%, 58%, 70%) and centrifuged for 17 h at 80,000 ×*g*. Eleven fractions were collected from the top of the gradient and analyzed by Western blot. As shown in Fig. 3A, internal membranes were found in fractions with lower density, while plasma membrane localized in the last fractions, consistently with previously reported data [46].

Results obtained from cellular fractionation showed that Ras2 and Cdc25 were mainly localized on internal membranes, while a consistent but minor fraction (~10%) of these proteins was localized at the plasma membrane level (Fig. 3B). Moreover, Cyr1 and Ira proteins showed the same pattern in fractionation experiments, supporting the hypothesis that the entire complex resides mostly on internal membranes. Only Ras2 and Ira proteins, but not adenylate cyclase and Cdc25, seem to reveal a concentration peak also in the lighter fractions, likely corresponding to mitochondria, according to fluorescence data, and consistently with what was already reported for Ras2 [23].

Table 2
Oligonucleotides used in this work.

Oligonucleotide	Sequence
IRA1HA FOR	AAGATTAGCAACAATGATACTGGCAAGAATGTCGTGCTCCCGGCCGCATGGATCCTATCC
IRA1HA REV	GGAAAAACGTAATAATCACTGCAATACTCTAATTTAAAAGGATGGCGGCGTTAGTATCG
IRA2HA FOR	AATAATGACAATG ATTTTATCAAGGATGTCATGTTCTGCTCGGCCGCATGGATCCTATCC
IRA2HA REV	ATAGATATTGATATTTCTTTCATTAGTTTATGTAACACCTGGATGGCGGCGTTAGTATCG

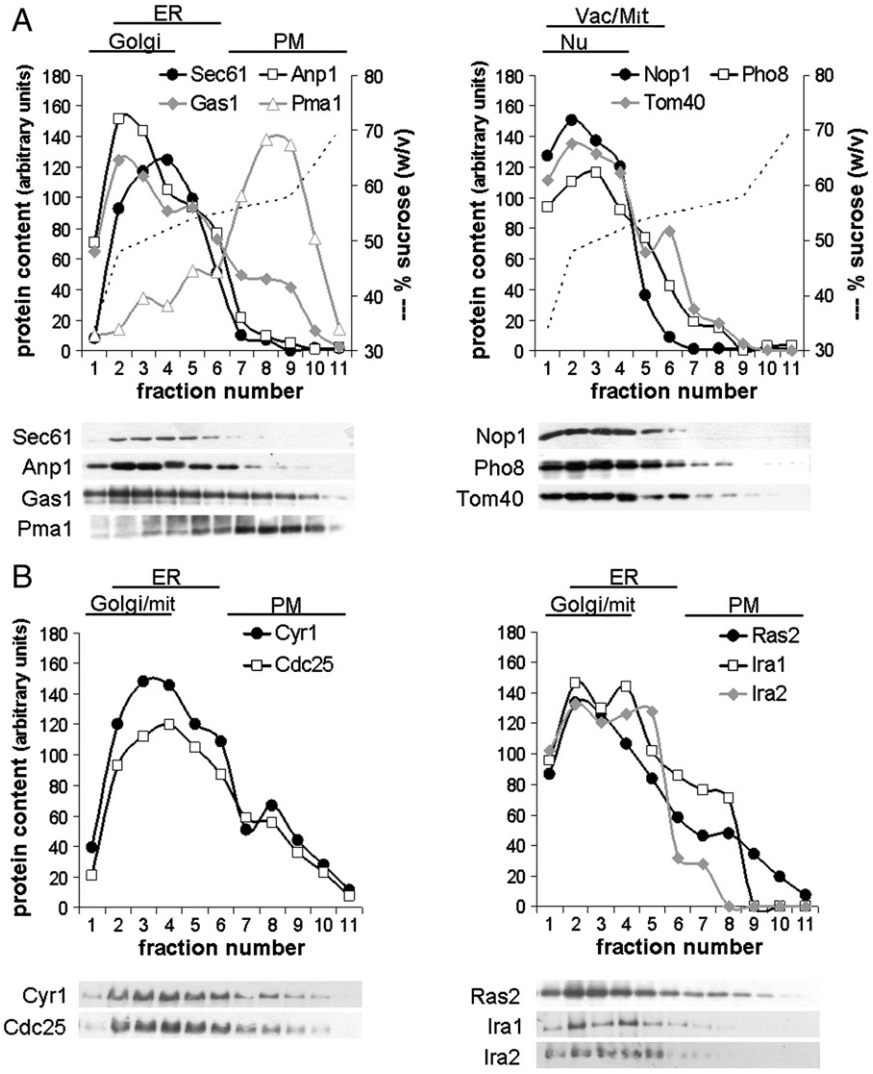

Fig. 3. Ras signaling complex mainly resides on internal membranes. Membranes were prepared from OL568-1C or YLR378C strain, then they were layered on sucrose gradient and centrifuged at 80,000 ×*g* for 17 h. 1-ml fractions were collected from the top of the gradient. Proteins were detected by Western blot. (A) Membrane marker distribution: Sec61 (ER), Anp1 (Golgi), Gas1 (ER, Golgi and plasma membrane), Pma1 (plasma membrane), Nop1 (nucleus), Pho8 (vacuolar membrane), Tom40 (mitochondria); (B) signaling transduction complex component localization. A typical distribution of the indicated proteins is shown out of at least four replicates. No differences were observed in marker distributions in different background strains.

The plasma membrane containing fractions were identified by visualizing mature Pma1. The analysis of the amount of protein present in the plasma membrane fractions revealed that the components of the Ras/adenylate cyclase complex are nonetheless significantly represented in the plasma membrane in exponentially growing cells, showing a ratio plasma membrane/internal membranes comparable to what was revealed for Gas1 protein, which has a complex internal and plasma membrane distribution [36], suggesting that in this physiological condition they could exert their essential function both on internal membranes and/or on the plasma membrane.

Since Cdc25 was previously shown to be efficiently imported and retained in the nuclear compartment [24], cellular fractionation was also performed on the W303-CDC25^{NES} strain, where Cdc25 was modified by the insertion of a strong Nuclear Export Signal, and is therefore excluded from the nucleus. This mutant Cdc25 protein has a distribution in internal membrane not different from the wild type protein (data not shown); besides, the nuclear marker, Nop1, partially overlays on the ER distribution (Fig. 3A). This suggests that on this gradient the nuclear envelope is not distinguishable from the ER membranes, and that the localization of Cdc25^{NES} protein in cellular membranes is not significantly different from the wild-type Cdc25. Unfortunately, the resolution of intracellular membrane compartments in the sucrose gradients in such conditions is too low to avoid partial overlapping of the different fractions. In order to better resolve the internal membranes, cellular fractionation was performed changing buffer compositions, replacing EDTA by 2 mM $MgCl_2$ in all solutions. In fact, in the presence of $MgCl_2$, the endoplasmic reticulum has been reported to have much higher density than Golgi membranes, likely because a Mg^{+}-dependent association of ribosomes with rough ER is preserved [46]. In these experimental conditions, most of the ER membranes were localized in the last gradient fractions corresponding to higher sucrose concentrations (Fig. 4A), dragging a minor quote of the other cellular membranes to the bottom, too. Ras2, Cyr1, Cdc25 and Ira proteins were also massively shifted to the bottom of the gradient, and a minor percentage could be observed in the middle, confirming that these proteins are mainly associated with ER membranes. Ras2 and Ira proteins were still found also in low density membranes, containing mitochondrial membranes (Figs. 3B and 4B), confirming previously reported data for Ras [23] and here for Ira2 (Fig. 2). The association of a minor fraction of Cyr1 with mitochondria could not be clearly revealed with this technique.

Both Ras2 and Cdc25 were recently reported to be affected by nutrient availability or by the hyper-activity of a PKA catalytic subunit, Tpk1. In order to investigate if Cyr1 and Ira2 protein localization

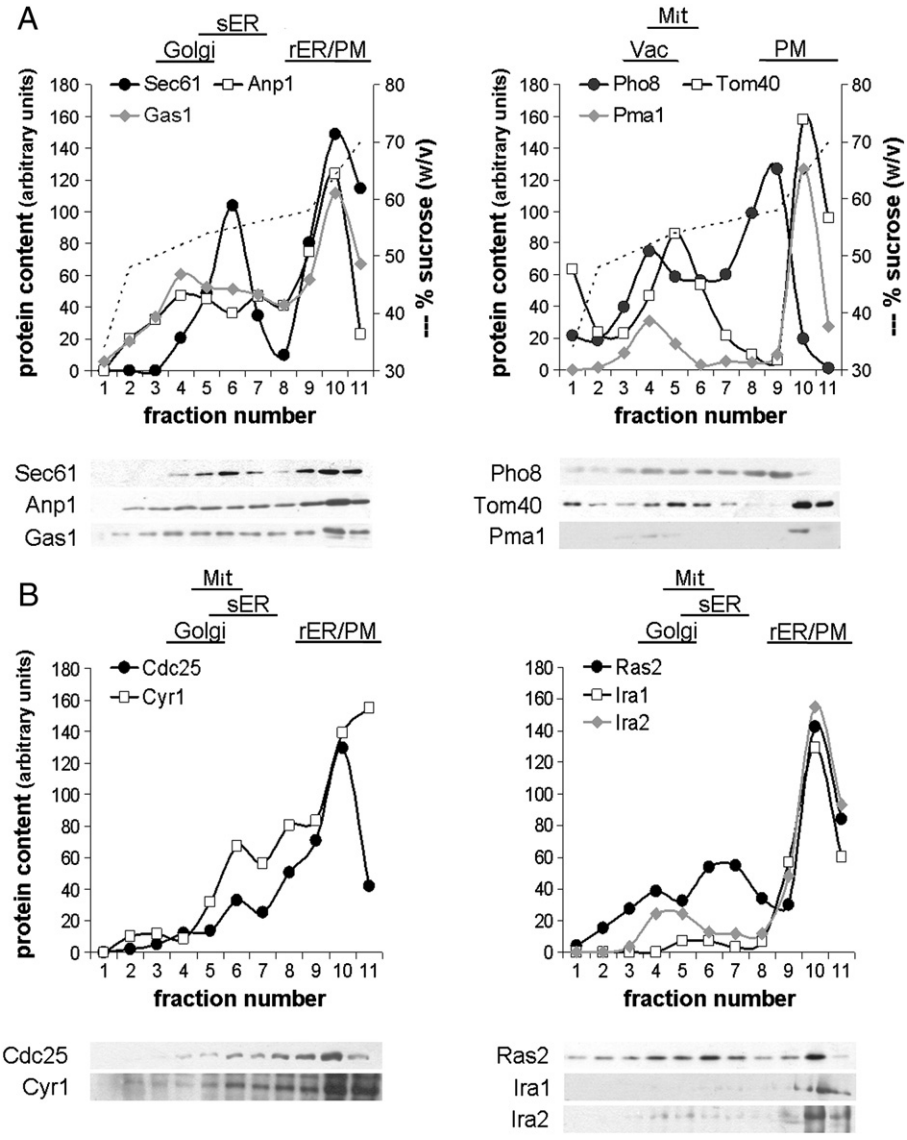

Fig. 4. Ras signaling complex is mainly localized on the ER membranes. 2 mM $MgCl_2$ replaced EDTA in all fractionation buffers. (A) Membrane marker distribution; B) signaling transduction complex component localization.

was also sensitive to such regulation, RT1230 and RT1240 strains were observed during exponential growth on glucose and after nutrient starvation in MES buffer (100 mM, pH 6.5) for 4 h. No changes were observed in either Cyr1 or Ira2 localization in ER membranes, where both the proteins were still observed in all tested conditions and in almost all of the cells observed. Nor did nutrient starvation affect localization of Cyr1 or Ira2 in mitochondria (data not shown).

To investigate if Cyr1 and Ira2 protein localization was sensitive to PKA hyper-activity, RT1210 and RT1220 strains were sporulated and the haploid strains expressing Cyr1-GFP or Ira2-GFP fusion proteins as unique version of the protein were transformed with an episomic plasmid (YEpTPK1) carrying *TPK1* gene. Co-localization with ER membranes was observed by co-transforming the strain with the YIplac204-T/C-Hmg1DsRED plasmid, and co-localization with mitochondria with *in vivo* DAPI staining. Tpk1 overexpression did not affect Cyr1 localization in ER or in the mitochondria (data not shown), but while co-localization with ER membranes was observed in all the cells expressing Ira2-GFP protein, either carrying YEpTPK1 plasmid or not, the overexpression of Tpk1 PKA subunit made Ira2-GFP co-localization with mitochondria much less evident, so that it was detectable in 40% of the cells, *versus*

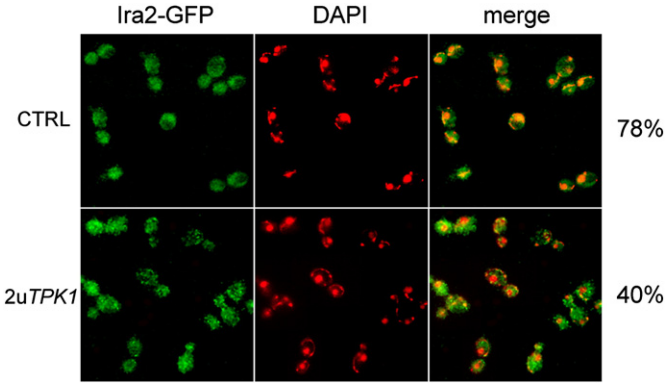

Fig. 5. Overexpression of PKA catalytic subunit Tpk1 counteracts Ira2 localization in mitochondria, driving it to ER membranes. Ira2 localization was observed in RT1211 (CTRL) and in RT1211 transformed with YEpTPK1 plasmid (2u*TPK1*), and the fraction of cells where co-localization of Ira2-GFP and DAPI-stained mitochondria was evident was calculated on at least 300 cells and indicated in the panel. Cells from different microscope fields were mounted in these panels to show representative patterns observed in the population.

the 78% in cells with physiological PKA activity showing evident Ira2-GFP localization in the mitochondria (Fig. 5).

Localization of Ras signaling complex in different compartments could be an effect of or a mechanism for feedback regulation by PKA, and could in its turn have effects on PKA pathway activation.

In order to analyze if Cdc25 nuclear export affects PKA activity, W303-CDC25^{NES} strain phenotype traits related to PKA activity were further investigated: no difference was found in glycogen accumulation in stationary phase (data not shown) or in growth rate and percentage of budded cells on glucose, but W303-CDC25^{NES} strain revealed a smaller cell size both on glucose (Fig. 6A) and on galactose (data not shown), and a higher heat shock resistance during exponential growth on glucose (Fig. 6B), which suggests a reduction in PKA activity in this strain. This is consistent with the phenotype recently reported for a strain carrying a double mutation in Cdc25 coding sequence, which substitutes Ser806 and Ser807 with glutamic acid. This mutant protein is no longer localized in the nuclear compartment, and shows phenotype traits typical of PKA-activity down-regulation [25].

Furthermore, the physiological relevance of the localization of the bulk of Ras signaling complex components in the ER membranes could be related to an active role of ER membranes in Ras signaling, or the complex could simply be inactive on ER membranes and represent a storage for Ras signaling components, where to drop them when the signal has to be reduced. In order to address this question, the presence of active Ras on ER membranes was analyzed by taking advantage of a Ras-GTP specific probe based on the Ras-binding domain of Raf-1, fused to GFP. Observation of cells expressing EGFP-RBD-3 probe and co-transformed with the YIplac204/TKC-DsRED-HDEL plasmid allowed to observe that during exponential growth on glucose active Ras2 is present mainly in the nuclear compartment besides in the plasma membrane, as previously reported [26], while an evident localization of the probe in the ER was difficult to evidence (data not shown) suggesting that Ras2-GTP is only marginally present in ER membranes. However, an active signaling complex, or at least an endogenous exchange activity, can be detected on internal membranes: proteins isolated in gradient fractions 1 to 5, corresponding to internal membranes, were incubated for 15 min either with 1 mM GDP or GTP, and then Ras2-GTP was purified by pull-down assay and visualized by Western blot. In all the analyzed fractions, the amount of purified Ras2-GTP was higher in the presence of GTP than in the presence of GDP, suggesting that an exchange activity exists on these membranes. The guanine nucleotide exchange on Ras2 was a Cdc25-dependent process since the GTP loading on Ras2 in a *cdc25*Δ YepTPK1 strain was abolished (Fig. 7).

4. Discussion

The reported results indicate a largely intracellular localization of Ras signaling complex: in fact, the bulk of Cyr1, Ras, Cdc25 and Ira proteins, examined both by fluorescence tagging and/or cellular fractionation, co-localized with internal membranes, while only a consistent but minor amount of these proteins was found associated with the plasma membrane fractions in exponentially growing cells. Moreover, the membrane fractionation performed in the presence of $MgCl_2$ demonstrated that these proteins are mainly associated to ER membranes; only Ras2 and Ira proteins appear to be also significantly present in lighter membranes, likely mitochondria. This is in agreement with fluorescence tagging experiments, which in turn reveal that also a minor fraction of Cyr1 protein is present in this last compartment. A mitochondrial localization was previously reported only for Ras2 [23], and it is consistent with the preliminary identification of Ira1 in the mitochondrial proteome by two distinct high-throughput approaches [47,48], while this is the first report stating the presence of Cyr1 in mitochondria. None of these proteins show any consensus for mitochondrial localization signals, so they probably associate to the mitochondria following a non-classical pathway. Consistently, also Ras targeting to the plasma membrane does not require the classical secretion pathway, while it requires endosomal and vacuolar membrane fusion class C *VPS* proteins and mitochondrial functionality [23].

Though Ras2 localization on internal membranes is not surprising, since it was already reported [20,27] and, trivially, enzymes required for Ras2 post-translational modifications were found on ER membranes

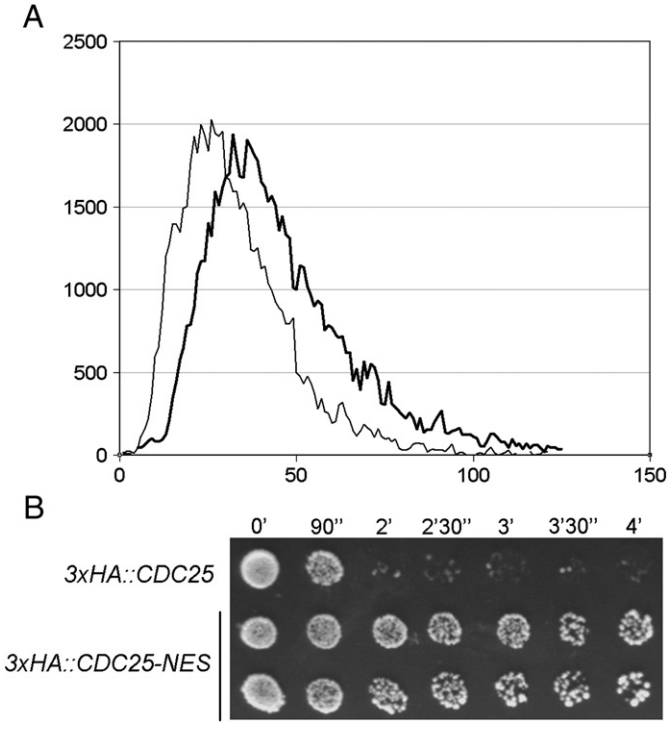

Fig. 6. Cdc25^{NES} strain shows features associated to a down-regulation in PKA activity. OL568-1C (W303 with *3xHA::CDC25*) (heavy line) and W303-CDC25^{NES} strain (thin line) cells exponentially growing in YPD medium were analyzed for cell volume with a Coulter Counter associated Channelyzer (panel A) and 10^4 cells were spotted on fresh YPD medium after being exposed to 51 °C for the indicated time (panel B).

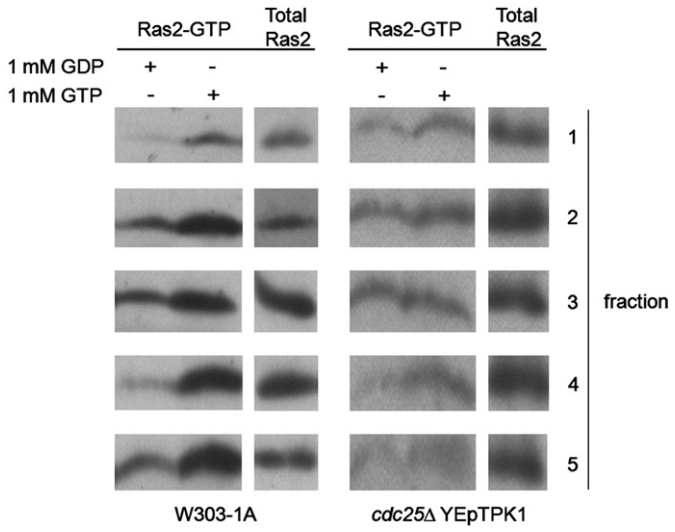

Fig. 7. GDP/GTP exchange can be detected in internal membranes. Membranes were extracted from YPD medium exponentially growing cells of W303-1A or *cdc25*Δ YEp*TPK*1 strains and fractionated on a sucrose gradient. Proteins from the fractions 1 to 5 were incubated with 1 mM GDP or GTP for 15 min and then with GST–RBD prebound to glutathione–Sepharose. Purified Ras2-GTP and total Ras2 protein were detected by Western blot using anti Ras2 antibodies (Santa Cruz Biotechnology).

[21], the findings that its effector Cyr1, and its regulative factors, Cdc25 and Ira proteins, are also localized on internal membranes suggest that Ras2 localization could have a functional meaning and it would not merely be linked to a post-translational modification process. Consistently, mammalian Ras isoforms, H-Ras, N-Ras and K-Ras, are found associated not only to plasma membrane but also to intracellular membranes: many studies have shown that in other organisms Ras proteins can control different pathways by acting in distinct cellular compartments such as plasma membrane, endosomes, Golgi or mitochondria [49–51]. In yeast, Ras signaling is important for several cellular pathways (PKA activity, filamentous or invasive growth), but up-to-now no clear evidence exists that different pools of Ras or Ras regulating proteins could be differently involved in these different signals, beyond data suggesting that Ras2 uses the ER as a signaling platform from which it negatively regulates the first step in the production of GPI-anchors for cell surface proteins [52].

Shuttling of Ras2 and Cdc25 from plasma membrane to internal membranes was reported to be connected to PKA activity and nutritional conditions, as well as control of nuclear localization of Cdc25 [25,27], while Cyr1 protein was reported to be more soluble in mutants with PKA hyper-activity [8]. Observation of fluorescence tagged Ira2 and Cyr1 proteins did not evidence any difference in cells growing on carbon sources different from glucose or after nutrient starvation (data not shown), suggesting that this nutritional regulation is a specific feature of Ras2 and Cdc25 localization. Though, PKA activity is involved in driving Ira2 away from the mitochondria (Fig. 5), as well as Cdc25 out of the nucleus and both Ras2 and Cdc25 away from the plasma membrane [25]. Driving all of these proteins to the ER compartments in case of PKA hyper-activity is expected to have a functional meaning.

Bhattacharya et al. [53] have suggested that *S. cerevisiae* Ras2 signaling function is confined to plasma membrane level, while normal cell growth does not require Ras2 attachment to plasma membrane. In fact, the mutant $Ras2^{C318S}$, that is not palmitoylated and subsequently not able to bind to the plasma membrane, supports a normal mitotic growth but fails to induce cAMP increase in response to glucose. It was suggested that Ras would sense the energetic condition within the cell [54], or the level of some glycolytic intermediates; none of these signals requires the localization of this complex on the plasma membrane. Anyway, the presence on the plasma membrane of another adenylate cyclase activating complex should also be considered: it is assessed that both the activating systems, Gpr1/Gpa2 and Ras complex, are required to cooperate for rapid response to glucose re-addition to glucose-starved or derepressed cells, while during exponential growth on glucose this is not necessary. In fact, it was reported that PKA hyper-activation causes Ras2 relocalization from plasma membrane to cytoplasm [27], while GPCR complex is unable to activate adenylate cyclase in the absence of active Ras, although very small amounts of active Ras are sufficient for GPCR system to activate glucose-induced transient cAMP spike [55]. A working model could be that during exponential growth PKA activity is finely tuned according to the nutritional condition by Ras complex working on internal membranes, while in the absence of glucose a higher fraction of Ras proteins and Cdc25 RasGEF are re-located to the membrane to cooperate with GPCR system and to achieve the fast and transient cAMP signal necessary to rapidly adapt to an incoming glucose availability. Accordingly, active Ras and an exchange activity can be detected on ER membranes during exponential growth on glucose (Fig. 7).

Mitochondrial membranes might represent another platform for Ras signaling. Phosphorylation-induced relocalization of mammalian K-Ras to endomembranes has been demonstrated to be associated with induction of apoptosis [50]. In yeast, the shutdown of cAMP–PKA signaling activity in wild-type cells involves targeting of Ras2p to the vacuole for proteolysis. Cells lacking Whi2p function exhibit an aberrant accumulation of activated Ras2 at the mitochondria in response to nutritional depletion, inducing actin-mediated apoptosis as a result of inappropriate Ras–cAMP–PKA activity [26]. Further work will be needed to clarify if mitochondrial Ras activity is specifically required for apoptosis induction.

Moreover, nuclear localization of Cdc25, Ira1 and active Ras2 suggests that nuclear compartment too could have a role in Ras signaling. It is well-known that PKA subunits are localized in the nucleus [56,57], and the interaction among these proteins in the nuclear compartment should be considered. For example, Cdc25 export from the nucleus is controlled by PKA activity, but on the other hand the exclusion of Cdc25 from the nuclear compartment is sufficient to affect PKA activation ([25] and this work).

Acknowledgements

We thank M. Jacquet, M. Vai, J. Winderickx, S. Munro, T. Endo, P. Coccetti, P. Branduardi, B. Glick, and JF. Cannon for the generous gift of strains, plasmids or antibodies, and M. Ceriani for technical assistance in confocal microscopy. This work was partially supported by UNICELLSYS to EM.

References

[1] J. Thevelein, J. de Winde, Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*, Mol. Microbiol. 33 (1999) 904–918.
[2] M. Baroni, E. Martegani, P. Monti, L. Alberghina, Cell size modulation by CDC25 and RAS2 genes in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 9 (1989) 2715–2723.
[3] M. Nakafuku, T. Obara, K. Kaibuchi, I. Miyajima, A. Miyajima, H. Itoh, S. Nakamura, K. Arai, K. Matsumoto, Y. Kaziro, Isolation of a second yeast *Saccharomyces cerevisiae* gene (GPA2) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1374–1378.
[4] S. Colombo, P. Ma, L. Cauwenberg, J. Winderickx, M. Crauwels, A. Teunissen, D. Nauwelaers, J. de Winde, M. Gorwa, D. Colavizza, J. Thevelein, Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*, EMBO J. 17 (1998) 3326–3341.
[5] T. Toda, I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, M. Wigler, In yeast, RAS proteins are controlling elements of adenylate cyclase, Cell 40 (1985) 27–36.
[6] S. Jones, M. Vignais, J. Broach, The CDC25 protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to ras, Mol. Cell. Biol. 11 (1991) 2641–2646.
[7] K. Tanaka, M. Nakafuku, F. Tamanoi, Y. Kaziro, K. Matsumoto, A. Toh-e, IRA2, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein, Mol. Cell. Biol. 10 (1990) 4303–4313.
[8] M. Mitts, D. Grant, W. Heideman, Adenylate cyclase in *Saccharomyces cerevisiae* is a peripheral membrane protein, Mol. Cell. Biol. 10 (1990) 3873–3883.
[9] J. Field, J. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. Wilson, R. Lerner, M. Wigler, Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method, Mol. Cell. Biol. 8 (1988) 2159–2165.
[10] W. Heideman, G. Casperson, H. Bourne, Adenylyl cyclase in yeast. Hydrodynamic properties and activation by trypsin. J. Biol. Chem. 262 (1987) 7087–7091.
[11] W. Heideman, G. Casperson, H. Bourne, Adenylyl cyclase in yeast: antibodies and mutations identify a regulatory domain, J. Cell. Biochem. 42 (1990) 229–242.
[12] N. Suzuki, H. Choe, Y. Nishida, Y. Yamawaki-Kataoka, S. Ohnishi, T. Tamaoki, T. Kataoka, Leucine-rich repeats and carboxyl terminus are required for interaction of yeast adenylate cyclase with RAS proteins, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8711–8715.
[13] F. Shima, Y. Yamawaki-Kataoka, C. Yanagihara, M. Tamada, T. Okada, K. Kariya, T. Kataoka, Effect of association with adenylyl cyclase-associated protein on the interaction of yeast adenylyl cyclase with Ras protein, Mol. Cell. Biol. 17 (1997) 1057–1064.
[14] K. Mintzer, J. Field, The SH3 domain of the *S. cerevisiae* Cdc25p binds adenylyl cyclase and facilitates Ras regulation of cAMP signalling. Cell. Signal. 11 (1999) 127–135.
[15] M. Mitts, J. Bradshaw-Rouse, W. Heideman, Interactions between adenylate cyclase and the yeast GTPase-activating protein IRA1, Mol. Cell. Biol. 11 (1991) 4591–4598.
[16] M. Vanoni, M. Vavassori, G. Frascotti, E. Martegani, L. Alberghina, Overexpression of the CDC25 gene, an upstream element of the RAS/adenylyl cyclase pathway in *Saccharomyces cerevisiae*, allows immunological identification and characterization of its gene product, Biochem. Biophys. Res. Commun. 172 (1990) 61–69.
[17] H. Garreau, M. Geymonat, G. Renault, M. Jacquet, Membrane-anchoring domains of Cdc25p, a *Saccharomyces cerevisiae* ras exchange factor, Biol. Cell 86 (1996) 93–102.
[18] G.E. Wheeler, A. Schibeci, R.M. Epand, J.B. Rattray, D.K. Kidby, Subcellular localization and some properties of the adenylate cyclase activity of the yeast, *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 372 (1974) 15–22.
[19] O. Rocks, A. Peyker, P. Bastiaens, Spatio-temporal segregation of Ras signals: one ship, three anchors, many harbors, Curr. Opin. Cell Biol. 18 (2006) 351–357.
[20] A.K. Sobering, M.J. Romeo, H.A. Vay, D.E. Levin, A novel Ras inhibitor, Eri1, engages yeast Ras at the endoplasmic reticulum, Mol. Cell. Biol. 23 (2003) 4983–4990.

[21] X. Dong, D. Mitchell, S. Lobo, L. Zhao, D. Bartels, R. Deschenes, Palmitoylation and plasma membrane localization of Ras2p by a nonclassical trafficking pathway in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 23 (2003) 6574–6584.
[22] L. Zhao, S. Lobo, X. Dong, A. Ault, R. Deschenes, Erf4p and Erf2p form an endoplasmic reticulum-associated complex involved in the plasma membrane localization of yeast Ras proteins, J. Biol. Chem. 277 (2002) 49352–49359.
[23] G. Wang, R. Deschenes, Plasma membrane localization of Ras requires class C Vps proteins and functional mitochondria in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 26 (2006) 3243–3255.
[24] R. Tisi, F. Belotti, C. Paiardi, F. Brunetti, E. Martegani, The budding yeast RasGEF Cdc25 reveals an unexpected nuclear localization, Biochim. Biophys. Acta 1783 (2008) 2363–2374.
[25] F. Belotti, R. Tisi, C. Paiardi, S. Groppi, E. Martegani, PKA-dependent regulation of Cdc25 RasGEF localization in budding yeast, FEBS Lett. 585 (2011) 3914–3920.
[26] J. Leadsham, K. Miller, K. Ayscough, S. Colombo, E. Martegani, P. Sudbery, C. Gourlay, Whi2p links nutritional sensing to actin-dependent Ras–cAMP–PKA regulation and apoptosis in yeast, J. Cell Sci. 122 (2009) 706–715.
[27] J. Dong, X. Bai, The membrane localization of Ras2p and the association between Cdc25p and Ras2–GTP are regulated by protein kinase A (PKA) in the yeast *Saccharomyces cerevisiae*, FEBS Lett. 585 (2011) 1127–1134.
[28] A. Wach, A. Brachat, R. Pöhlmann, P. Philippsen, New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*, Yeast 10 (1994) 1793–1808.
[29] F. Tripodi, V. Zinzalla, M. Vanoni, L. Alberghina, P. Coccetti, In CK2 inactivated cells the cyclin dependent kinase inhibitor Sic1 is involved in cell-cycle arrest before the onset of S phase, Biochem. Biophys. Res. Commun. 359 (2007) 921–927.
[30] C. Reinke, P. Kozik, B. Glick, Golgi inheritance in small buds of *Saccharomyces cerevisiae* is linked to endoplasmic reticulum inheritance, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 18018–18023.
[31] A. Toulmay, W.A. Prinz, A conserved membrane-binding domain targets proteins to organelle contact sites, J. Cell Sci. 125 (2012) 49–58.
[32] S. Colombo, D. Ronchetti, J. Thevelein, J. Winderickx, E. Martegani, Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*, J. Biol. Chem. 279 (2004) 46715–46722.
[33] F. Sidoux-Walter, N. Pettersson, S. Hohmann, The *Saccharomyces cerevisiae* aquaporin Aqy1 is involved in sporulation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 17422–17427.
[34] L. Popolo, M. Vai, The Gas1 glycoprotein, a putative wall polymer cross-linker, Biochim. Biophys. Acta 1426 (1999) 385–400.
[35] M.R. Koch, L. Pillus, The glucanosyltransferase Gas1 functions in transcriptional silencing, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11224–11229.
[36] W.K. Huh, J.V. Falvo, L.C. Gerke, A.S. Carroll, R.W. Howson, J.S. Weissman, E.K. O'Shea, Global analysis of protein localization in budding yeast, Nature 425 (2003) 686–691.
[37] J. Jungmann, S. Munro, Multi-protein complexes in the cis Golgi of *Saccharomyces cerevisiae* with alpha-1,6-mannosyltransferase activity, EMBO J. 17 (1998) 423–434.
[38] C. Meisinger, T. Sommer, N. Pfanner, Purification of *Saccharomyces cerevisiae* mitochondria devoid of microsomal and cytosolic contaminations, Anal. Biochem. 287 (2000) 339–342.
[39] D. Klionsky, S. Emr, Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase, EMBO J. 8 (1989) 2241–2250.
[40] J. Fuchs, J. Loidl, Behaviour of nucleolus organizing regions (NORs) and nucleoli during mitotic and meiotic divisions in budding yeast, Chromosome Res. 12 (2004) 427–438.
[41] R. Serrano, Structure, function and regulation of plasma membrane H(+)-ATPase, FEBS Lett. 325 (1993) 108–111.
[42] R. Serrano, M.C. Kielland-Brandt, G.R. Fink, Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+-and Ca2+-ATPases, Nature 319 (1986) 689–693.
[43] A. Pierleoni, P.L. Martelli, P. Fariselli, R. Casadio, BaCelLo: a balanced subcellular localization predictor, Bioinformatics 22 (2006) e408–e416.
[44] O. Emanuelsson, S. Brunak, G. von Heijne, H. Nielsen, Locating proteins in the cell using TargetP, SignalP and related tools, Nat. Protoc. 2 (2007) 953–971.
[45] A. Franzusoff, K. Redding, J. Crosby, R.S. Fuller, R. Schekman, Localization of components involved in protein transport and processing through the yeast Golgi apparatus, J. Cell Biol. 112 (1991) 27–37.
[46] K. Roberg, N. Rowley, C. Kaiser, Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*, J. Cell Biol. 137 (1997) 1469–1482.
[47] A. Sickmann, J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. Meyer, B. Schönfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, C. Meisinger, The proteome of *Saccharomyces cerevisiae* mitochondria, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13207–13212.
[48] J. Reinders, R. Zahedi, N. Pfanner, C. Meisinger, A. Sickmann, Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics, J. Proteome Res. 5 (2006) 1543–1554.
[49] V. Chiu, T. Bivona, A. Hach, J. Sajous, J. Silletti, H. Wiener, R.N. Johnson, A. Cox, M. Philips, Ras signalling on the endoplasmic reticulum and the Golgi. Nat. Cell Biol. 4 (2002) 343–350.
[50] T. Bivona, S. Quatela, B. Bodemann, I. Ahearn, M. Soskis, A. Mor, J. Miura, H. Wiener, L. Wright, S. Saba, D. Yim, A. Fein, I. Pérez de Castro, C. Li, C. Thompson, A. Cox, M. Philips, PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis, Mol. Cell 21 (2006) 481–493.
[51] E. Chang, M. Philips, Spatial segregation of Ras signaling: new evidence from fission yeast, Cell Cycle 5 (2006) 1936–1939.
[52] A.K. Sobering, R. Watanabe, M.J. Romeo, B.C. Yan, C.A. Specht, P. Orlean, H. Riezman, D.E. Levin, Yeast Ras regulates the complex that catalyzes the first step in GPI-anchor biosynthesis at the ER, Cell 117 (2004) 637–648.
[53] S. Bhattacharya, L. Chen, J. Broach, S. Powers, Ras membrane targeting is essential for glucose signaling but not for viability in yeast, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 2984–2988.
[54] S. Rudoni, S. Colombo, P. Coccetti, E. Martegani, Role of guanine nucleotides in the regulation of the Ras/cAMP pathway in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1538 (2001) 181–189.
[55] C. Paiardi, F. Belotti, S. Colombo, R. Tisi, E. Martegani, The large N-terminal domain of Cdc25 protein of the yeast *Saccharomyces cerevisiae* is required for glucose-induced Ras2 activation, FEMS Yeast Res. 7 (2007) 1270–1275.
[56] G. Griffioen, J. Thevelein, Molecular mechanisms controlling the localisation of protein kinase A, Curr. Genet. 41 (2002) 199–207.
[57] V. Tudisca, V. Recouvreux, S. Moreno, E. Boy-Marcotte, M. Jacquet, P. Portela, Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA subunits under different growth conditions, Eur. J. Cell Biol. 89 (2010) 339–348.
[58] B. Thomas, R. Rothstein, Elevated recombination rates in transcriptionally active DNA, Cell 56 (1989) 619–630.
[59] M. Geymonat, L. Wang, H. Garreau, M. Jacquet, Ssa1p chaperone interacts with the guanine nucleotide exchange factor of ras Cdc25p and controls the cAMP pathway in *Saccharomyces cerevisiae*, Mol. Microbiol. 30 (1998) 855–864.